Author(s): Sipponen, Mika Henrikki & Lapierre, Catherine & Méchin, Valérie & Baumberger, Stéphanie

Title: Isolation of structurally distinct lignin carbohydrate fractions from maize stem by sequential alkaline extractions and endoglucanase treatment

Year: 2013

Version: Pre-print

Isolation of structurally distinct lignin-carbohydrate fractions from maize stem by sequential alkaline extractions and endoglucanase treatment

Mika Henrikki Sipponen[a,b]*, Catherine Lapierre[b], Valerie Méchin[b], Stéphanie Baumberger[b]

[a]Aalto University, School of Chemical Technology, Department of Biotechnology and Chemical Technology, Espoo, Finland

[b]Institut Jean-Pierre Bourgin, UMR 1318 INRA/AgroParisTech, Pôle SCSM, 78000 Versailles, France

* Corresponding author. Tel.: +358947022547, Fax: +3589462373, Postal address: PO BOX 16100 FI-00076 Aalto, E-mail: mika.sipponen@aalto.fi

Abstract

Sequential fractionation of extractive-free maize stems was carried out using two mild alkaline extractions (0.5 M and 2 M NaOH, 20°C, 24 h) before and after endoglucanase treatment. This procedure provided two lignin-carbohydrate fractions (LC1 and LC2) recovered after each alkali treatment. LC1 and LC2 contained 39% and 8% of the total lignin amount, respectively. These two fractions contained structurally distinct lignin molecules. While the content of resistant interunit bonds in lignin was 77% in LC1, it was increased up to 98% in LC2. Not unexpectedly, both alkali-soluble fractions contained substantial amount of *p*-coumaric and ferulic acids ether-linked to lignins. These results outline heterogeneity of maize stem lignins related to fractionation of grass materials.

Keywords: Maize stem, fractionation, lignins, endoglucanase, lignocellulose

1 Introduction

Materials and energy based on renewable resources are sought to gradually replace those obtained from fossil sources and relying largely on crude oil refining. This change is driven both in the EU and in the US by socio-economic factors (Domac et al., 2005) as well as policy decisions (Charles et al., 2007) to increase the proportion of renewable components in transportation and energy sectors. Grasses represent a substantial reservoir of renewable lignocellulosic biomass that may be sustainably collected together with other agricultural residues as feedstock for so-called biorefineries with the scope of complete utilization of the cell wall components including lignins and hemicelluloses.

Grass lignins are made of syringyl (S) and guaiacyl (G) units together with lower amounts of *p*-hydroxyphenyl (H) units. These units are linked by labile β-O-4 bonds as well as by resistant interunit bonds, referred to as condensed bonds (Adler, 1977). Compared to non-grass cell walls, the organization of grass cell walls is more complex due to presence of ferulic acid (FA) that cross-links arabinoxylans (AX) and lignins (Grabber et al., 2000). Two other structural specificities of grass lignins are their acylation by *p*-coumaric acid (CA), mainly on S lignin units as reviewed in Ralph (2010) and high content of free phenolic groups as reviewed by Lapierre (2010). The latter structural trait makes half of total grass lignins easily soluble in alkali at room temperature (Lapierre et al., 1989), a property that is not shared by wood lignins (Beckman et al., 1923).

Maize (*Zea mays* L.) is a major crop that has frequently served as a model for fractionation and pretreatment of agricultural residues (corn stover and straw). Lignins make

approximately one-fifth of mature maize stems and should be addressed as valuable components rather than as low-value byproducts. Therefore, isolation of lignins should be carefully considered in the fractionation processes of lignocellulose biorefineries as lignin polymers isolated by mild procedures may be better suited for value-added applications such as enhancers of hydrophobic properties of renewable fibers (Sipponen et al., 2010). Isolation of carbohydrate-free technical lignins without severe structural changes and/or costly purification schemes remains a challenge as native lignins are covalently linked to hemicelluloses components (Gellerstedt and Henriksson, 2008). These covalent bonds are formed in the process of lignin polymerization when quinone methide intermediates spontaneously react with nucleophilic groups such as the carboxylic groups of uronic acids, the alcoholic groups of sugars or their hemiketal groups. Such reactions lead to ester, ether or glycosidic bonds between sugars and lignin units (Fengel and Wegener, 1983). Therefore, rather than attempting to isolate carbohydrate-free lignins, an alternative approach would be fractionating grass cell walls under mild conditions, which may allow co-solubilization of lignins together with carbohydrate and recovery of these lignin-carbohydrate fractions (LC) in which the structure of native lignins may be better retained.

For many decades, mild alkaline treatment of straw has been used to increase its feeding value (Jackson, 1977). Alkaline pretreatments seem also well suited to improve saccharification of grass materials in biotechnological conversion processes (Li et al., 2012). Further, endoglucanases (EGs) (EC 3.2.1.4) cleave specifically cellulose, and may thus allow isolation of a portion of lignins otherwise structurally restricted from dissolution in alkali. Previously, endoglucanase hydrolysis of cellulose has been reported prior to isolation of LCCs from wood and pulp materials (Lawoko et al., 2006). Furthermore,

EG treatment in series with alkaline extraction has been described for upgrading cellulose pulp into viscose (Ibarra et al., 2010). However, studies on utilization of EGs as tools in bioconversion of grass materials are scarce. In this study, a sequential alkaline extraction of maize stalk with the aid of EG treatment is presented in order to isolate, under mild conditions, lignin-hemicellulose fractions for structural analysis. Evidence is provided that two alkali-soluble LC fractions with quite distinct structural properties can be isolated from extractive-free and mature maize stems.

2 Materials and methods

Maize F2 (INRA) line was grown on experimental field conditions in Lusignan (France), harvested at silage stage, and stems without leaves ground to pass a 1 mm-screen exhaustively extracted by water and then 96% ethanol in a Soxhlet apparatus. The extractive-free (EFR) maize stem (MS) was recovered with a 54% yield (weight percentage on a dry matter basis). The endo-1,4-β-D-glucanase Novozym 476 was obtained from Novozymes (Denmark). This commercial preparation contained 6.7 mg mL^{-1} protein, assayed using the method of Bradford (1976).

2.1 Determination of Enzyme activities

Endoglucanase activity was determined according to Ghose (1987) using 1.5% (w/v) carboxymethyl cellulose (CMC) Na-salt, low viscosity (Sigma) as substrate. The assay for endoxylanase activity was adapted from Nakamura et al. (1993) using 1.5% (w/v) beechwood xylan (Sigma-Aldrich) or larchwood xylan (Ega-Chemie) suspension as substrate in 50 mM Na-citrate buffer (pH 4.8) at 50°C for 10 min. The amount of reduc-

ing sugars formed in the endoxylanase and endoglucanase assays were determined by the DNSA method (Miller, 1959) using xylose and glucose standards, respectively. One international unit of activity (IU) refers to the amount of the enzyme that releases one μmol of xylose or glucose in one minute under the assay conditions.

2.2 Isolation of alkali-soluble LCs

The LCs were isolated according to the scheme in Fig. 1. Maize EFR (17.5 g) was extracted with 360 mL of 0.5 M aqueous NaOH under N_2 in a glass bottle at 20°C for 24 h. The solid residue was separated from the alkaline extract by centrifugation, washed repeatedly with deionized water until neutral pH and lyophilized giving residue R1. The wash-supernatants were combined with the primary alkaline extract, and the solution was clarified by filtration (Whatman no. 4) before adjustment to pH 2 by 6 M HCl. The suspension was kept at +4°C in dark for 16 h before the precipitate was separated by centrifugation, suspension washed three times with deionized water acidified by HCl (pH 2), and lyophilized giving LC1. R1 (2% suspension w/v) was hydrolyzed with 52.8 IU g^{-1} (activity measured on CMC) of endo-1,4-β-D-glucanase preparation "Novozym 476" (Novozymes, Denmark) in 0.1 M Na-acetate buffer (pH 5) for 90 h at 45°C. Sodium azide (0.025% w/v) was used to prevent microbial contamination. The hydrolyzed solids were separated by centrifugation, washed copiously with deionized water, and lyophilized giving residue R2. Supernatant of the enzymatic hydrolysate was analyzed for its monosaccharide content before and after hydrolysis in 4% (w/w) H_2SO_4 at 121°C for 1 h. R2 was extracted with 2 M NaOH (4% w/v suspension) for 24 h at 20°C under N_2, and LC2 as well as residue R3 recovered as described above for the first alkaline extraction. Laboratory grinder (IKA) equipped with a blunt-edged blade was used to

disintegrate the compact freeze-dried residues. For calculation of mass balances, the lyophilisates were weighed immediately after freeze-drying and were considered as moisture-free. Dry matter contents of the lignocellulosic fractions were determined gravimetrically in parallel with each extraction step by drying separate samples over-night at 105°C.

2.3 Analytical procedures

2.3.1 Lignin determination and carbohydrate analysis

Lignin content was determined gravimetrically from the maize EFR and from the corresponding LC and residue samples according to the Klason protocol adapted from Dence (1992). Structural carbohydrates in residue samples were analyzed based on published protocol (Sluiter et al., 2011) scaled down to enable analysis of 15 mg samples in screw-cap glass tubes. Three different methods were compared for determination of carbohydrate compositions of the isolated LCs. They included (1) analysis of the filtrate recovered from Klason lignin determination, (2) hydrolysis of LCs in 4% (w/w) H_2SO_4 at 121°C for 1 h, and (3) hydrolysis of LCs in 2 M trifluoroacetic acid (TFA) at 120°C for 2 h (Blakeney et al., 1983). Monosaccharides in the endoglucanase hydrolysate as well as in the Novozym 476 preparation were analyzed before and after post-hydrolysis according to Sluiter et al. (2008). The difference between monosaccharides was interpreted as complex carbohydrate. Sugar recovery standards were used to correct for degradation of monosaccharides. Acid methanolysis was used to solubilize uronic acids (Willför et al., 2009). Briefly, 15 mg of EFR or LC samples were treated with 2 ml of 2 M anhydrous HCl in MeOH for 5 h at 100°C and the diluted methanolysates analyzed by high-performance anion-exchange chromatography (HPAEC) with a system consist-

ing of a CarboPac PA-1 column (guard column 4×50mm and analytical column 4×250 mm) (Dionex, Sunnyvale, CA, USA) coupled to a pulsed amperometric detector (Dionex ED 40). Injection volume was 25 µl, column temperature 20 °C, and galacturonic acid and glucuronic acid eluted by a linear gradient from 100 mM NaOH to 300 mM Na-acetate in 30 min at 1.0 mL min^{-1}. Neutral monosaccharides were analyzed by HPAEC-PAD system under similar conditions as described above, except that isocratic elution by 2.5 mM NaOH at 1.0 mL min^{-1} was performed. Calibration was performed using external standards ranging from 0.3 to 5.0 µg mL^{-1}, and the samples were diluted to give response within the calibration. All carbohydrate and lignin determinations were performed in duplicates.

2.3.2 Analysis of lignin structure by thioacidolysis

Thioacidolysis of 10-15 mg of the samples was carried out as previously described (Lapierre et al., 1995), using heneicosane C_{21} (Fluka) as internal standard (IS). Lignin-derived H, G and S thioacidolysis monomers were analyzed as their trimethylsilyl derivatives by gas chromatography-mass spectrometry (GC-MS). The quantitative determination of H, G and S monomers was performed from ion chromatograms reconstructed at m/z 239, 269 and 299, respectively, as compared to the IS signal measured from the ion chromatogram reconstructed at m/z (57+71+85). The molar yield of the thioethylated monomers released from the sample was calculated on the basis of its Klason lignin content. The analyses were performed in duplicates.

2.3.3 Determination of ester- and ether-bound *p*-hydroxycinnamic acids

Ester- and ether-linked *p*-coumaric acid (CA) and ferulic acid (FA) were determined by mild and severe alkaline hydrolysis, respectively, as described by Zhang et al (2011). Briefly, 50 mg of sample was subjected to mild alkaline hydrolysis (2 M NaOH, 5 mL, 20 h at room temperature) or treatment in severe alkaline conditions in teflon vials placed in an autoclave reactor (4 M NaOH, 5 mL, 2 h at 170°C). In both treatments, 1 mL ethyl vanillin (EtV) in MeOH (1 mg mL^{-1}) was added as IS before the reaction, and the phenolic acids were analyzed from neutralized hydrolysates after a precipitation procedure (Culhaoglu et al., 2011) by high-performance liquid chromatography coupled to a photodiode array detector (HPLC-PDA). Peak areas were integrated based on signal at 280 nm. The amount of ether-bound CA and FA was interpreted as the difference between the amounts of phenolic acids obtained from the severe and mild alkaline hydrolysis. Analyses were performed in duplicates.

2.3.4 ATR-FTIR spectroscopy

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra were recorded using Nexus 470 FT-IR (American Nicolet Company). Averaged spectra from 5-10 individual acquisitions of each sample with 10 scans were calculated.

3 Results and Discussion

The activities of a commercial endoglucanase Novozym 476 with respect to CMC and hardwood xylan were similar to the literature values (Lawoko, 2005). However, no activity on softwood xylan (larch) was detected. This data is given in Table S1 in the Elec-

tronic Annex. Two alkali-soluble lignin-carbohydrate (LC) fractions were recovered from the sequential and mild treatment of extractive-free maize stem with alkali (aqueous NaOH, 24 h room temperature) and with the commercial endoglucanase. The first fraction, LC1, recovered by a mild alkali treatment with 0.5 M NaOH followed by an acidification-induced precipitation step, accounted for 18% of the starting material (Table 1). Its Klason lignin (KL) content revealed that 39% of the total lignin amount was recovered in this LC1 fraction. The second fraction LC2, recovered after endoglucanase hydrolysis of the LC1-free residue, then 2 M NaOH alkali treatment, accounted only for 7.6% of the starting material and its lignin content accounted for 8.2% of the total lignin amount. The total mass of the recovered fractions (LC1+LC2+R3) accounted for 68% of the starting material. Their KL content accounted for 47% of the KL content of the starting material, which suggests loss of lignins during the fractionation procedure mainly due to incomplete precipitation after the alkaline extractions. In addition, the difference of DM yield (Table 1) includes 3.8% carbohydrate released by the endoglucanase treatment of R1. The LC and R fractions were analyzed with respect to lignins and carbohydrates as well as for CA and FA linked to wall polymers, with the objective to monitor the distribution of these grass cell wall components during the fractionation of maize stems.

3.1 Alkali fractionation of maize stems lead to fractions with distinct neutral sugar composition and lignin content

The maize EFR contained 17.3% Klason lignin and 63.2% total carbohydrate (Table 2), with a neutral sugar composition indicating the presence of 40.0 % cellulose (glucan) and 22.6 % pentosans. Galacturonic and glucuronic acids were detected in trace

amounts (< 0.1% w/w) after acid methanolysis suggesting their prior removal in the water-ethanol extraction of the maize stem material. Three different hydrolysis conditions were tested for the determination of carbohydrate content of the LC fractions. On the basis of the results given in supplementary data (Table S2 in the Electronic Annex), the highest carbohydrate content was achieved by 4% H_2SO_4 hydrolysis (1 h, 121°C). The lowest sugar content was obtained from the KL filtrate, probably due to sugar degradation in these more severe conditions. However, this procedure revealed higher glucan content in LC2 probably due to incomplete hydrolysis of cellulose in more severe hydrolysis conditions. Thus, for analysis of carbohydrate composition of LCs possibly containing cellulose, it may be necessary to use both the two-stage and dilute H_2SO_4 hydrolysis methods.

The compositional analysis of the solid residues and of the LC fractions (Table 2) indicated that the lignin content in the solid residues decreased at each LC alkali extraction step, concomitantly to the enrichment of lignin in the residue fractions. In contrast, the endoglucanase treatment lead to an increased lignin content from R1 (8.3%) to R2 (10.2%), in agreement with the selective release of carbohydrates into the endoglucanase hydrolysate (Table 3). As expected, the EG treatment mainly released cellulose fragments with glucose accounting for 80% of the total carbohydrate released from R1. Unexpectedly, 66% of the released glucan was in the free monosaccharide form. This result suggested, besides endoglucanase activity, presence of cellobiohydrolase and cellobiase in the enzyme preparation used since these activities are required for hydrolysis of cellulose to glucose. With respect to the mass balance, based on the carbohydrate analysis, 5.8% (w/w) of R1 was released as anhydrosugars by treatment with Novozym 476, compared to the 10.5% total DM removal calculated from mass balances (data not

shown). The difference may be resulting on one hand from sample losses and on the other from release of non-carbohydrate compounds such as lignin during the treatment.

As a whole, the sequential fractionation procedure lead to a lignin content reduction in the solid material by a factor 2.2 and a carbohydrate content increase by a factor 1.4 mainly by enrichment of cellulose (R3 compared to starting material). Not unexpectedly, the neutral sugar composition of the LC and R fractions indicated that hemicelluloses (arabinoxylans) were selectively extracted at each alkali treatment step leading to residues enriched in cellulose compared to the initial MS EFR material. Though LC2 exhibited higher carbohydrate content than LC1, their relative content of xylan was similar with respect to total carbohydrate (83%). The ratio of xylose to arabinose of LC1 (7.9) was close to the starting material MS EFR (8.6) whereas LC2 had higher xylose to arabinose ratio (13.9). The main differences in global compositions (Table 2) of the two LCs can be summarized as first, 2.5-times higher glucan content in LC2 compared to LC1. Second, LC1 contained two-times higher Klason lignin content (38.3%) compared to LC2 (18.7%). Thioacidolysis was performed to analyze structure of the lignins in the two LCs.

3.2 Thioacidolysis revealed contrasting lignin structures of LC1 and LC2

Thioacidolysis is an analytical degradation procedure that depolymerizes lignin by cleavage of the labile β-O-4 bonds that are major interunit bonds in native lignins (Lapierre, 2010). When expressed on the basis of the lignin content, the recovery yield of thioacidolysis monomers is therefore a close reflection of these parent structures in lignins. Conversely, this yield is decreased when the frequency of lignin units involved

in resistant interunit bonds, referred to as condensed linkages, is high. In agreement with previously published data (Méchin et al. 2000), the lignin-derived monomers released from the lignins of the extractive-free maize stem were obtained with a yield close to 800 µmol per gram lignin (Table 4). This yield means that about 17% of lignin units are only involved in labile β-O-4 bonds or, conversely, that 83% are involved in resistant linkages. While H monomers were obtained as trace components (about 1% of thioacidolysis monomers), G and S monomers were recovered in close relative amounts (52% G and 46% S). The comparison of R1 and LC1 with MS EFR indicated that the 0.5 M NaOH alkali treatment made some fractionation in native lignins. The lignins recovered in the LC1 were found to be slightly enriched in S units and in units only involved in β-O-4 bonds (54% and 23%; as compared to 46 and 17% in the initial sample).

The β-O-4 yield of LC1 accounted for 54% of the initial amount of the MS EFR, calculated using data in Table 1 and Table 4. This means that more than half of the MS EFR's lignins that contained units only involved in β-O-4 linkages were solubilized in 0.5 M NaOH and recovered in LC1. Comparison of R1 and R2 showed that the enzymatic hydrolysis resulted in slight enrichment of uncondensed lignins in R2. In contrast, the final 2 M alkali extraction of R2 further reduced by 42% the thioacidolysis yield compared to the final residue R3 with smaller S/G ratio (0.61) than in the initial material. The uncondensed lignins solubilized in 2 M NaOH were not recovered in LC2 by the precipitation procedure. Consequently, the two recovered LC fractions exhibited contrasted lignin profiles, with 77% of resistant interunit bonds in LC1 versus 98% in LC2. These two alkali-soluble fractions were further characterized by analyzing the amount and linkage types with respect to *p*-coumaric and ferulic acids.

3.3 Fractionation affected the binding modes and proportion of *p*-coumaric and ferulic acid in the isolated fractions

Alkaline hydrolyses carried out under mild and severe conditions were used to study ester- and ether-bound hydroxycinnamic acids in the isolated fractions. The phenolic compounds released by alkaline hydrolysis of each fraction are given in supplementary data (Table S3 in the Electronic Annex). It is noteworthy that the severe alkaline hydrolysis of the samples leads to generation of syringaldehyde and vanillin, probably due to degradative cleavage of S and G units of lignin, respectively. Though small amounts of syringaldehyde and vanillin were formed by degradation of lignin in severe hydrolysis conditions, CA and FA were the major phenolic constituents detected after mild and severe alkali hydrolysis of each fraction. Not unexpectedly, the ratio of ester- to ether-linked hydroxycinnamic acids in the lignocellulosic fractions decreased subsequently to each alkaline extraction due to hydrolysis of ester linkages (Table 5). The content of ester-linked CA observed in the current study for MS EFR is in agreement with literature value for F2 maize line (Méchin et al., 2000). By contrast, the LC or R fractions recovered after alkaline hydrolysis were found to be enriched in FA and CA units that resist mild alkaline hydrolysis. Their most likely bonding modes might be ether linkages. The initial amount of esterified CA related to the total CA observed by 4 M NaOH hydrolysis is 88%, which in accordance with earlier estimation that 90% of CA is esterified to lignin in wheat straw (Lawther et al., 1996). The higher proportion of ether-linked CA in LC1 than in LC2 suggests that CA is etherified to lignins, according to the two-fold higher lignin content of this fraction compared to LC2 and starting material MS EFR. The total amount of CA and FA in the unsolubilized fractions decreased grad-

ually from MS EFR to R3 as a result of the alkaline treatments. Interestingly, EG treatment of R1 decreased slightly content of ester-bound CA and FA in R2 (Table 5), which could be due to release of lignins and arabinoxylans associated with cellulose before its hydrolysis by the EG.

Based on mass balances of the initial 0.5 M NaOH extraction (data not shown) most of initial CA (75%) and FA (65%) were released as free acids in the first alkaline hydrolysate. Because 88% of the total amount of CA was ester-linked to lignins, the high proportion of solubilized lignins (68%) from MS EFR by the mild alkaline treatment, calculated from KL mass balances (Table 1), was probably resulting from increased content of phenolic hydroxyl groups in lignins. In turn, relatively low precipitation yield of LC1 compared to total amount of solubilized lignins suggested that these lignins are readily soluble even at pH 2. To compare fractionation of hydroxycinnamic acids into the main isolated fractions, mass balances of FA and CA are given in Table 6. Results show that 32% of FA was detected in the isolated fractions whilst only 17% of CA was recovered. FA units, which are etherified to lignin and ester-linked to hemicelluloses in grass cell walls have, in addition, been shown to oxidatively couple to dimers (Lapierre et al., 2001) making such structures less susceptible to alkaline hydrolysis. The initial proportion of ether-linked FA was five times higher than ether-linked CA in EFR (Table 5). Indeed, in contrast to low total yield of esterified hydroxycinnamic acids CA and FA (7% and 5%, respectively), majority of etherified CA and FA (91% and 81%, respectively) were recovered in the main fractions (Table 6). These points may in turn explain higher recovery and content of FA than CA in the isolated LCs. Moreover, majority of lignins in wheat straw have been suggested to be directly ether-linked to arabinose (Ara) moieties of arabinoxylans (Lawther and Sun, 1996). These ether-linkages would have

largely survived the alkaline extractions used in the current study, in contrast to ester-linked hydroxycinnamic acids (Table 5) as discussed above. However, since the LC fractions were not further fractionated or purified herein, no conclusion can be drawn regarding the quantity of covalent lignin-carbohydrate bonds.

3.4 ATR-FTIR spectroscopy was found suitable to monitor composition and structural changes of the maize stem samples along fractionation

Comparison of the ATR-FTIR spectra (Figure S1 in Electronic Annex) of the fractions in the 1900-600 cm^{-1} region was used to monitor the structural changes occurring along the sequential fractionation of maize stem. The spectra of untreated MS EFR exhibited broad bands at 900-1100 cm^{-1} with a strong band at 1030 cm^{-1} assignable to aromatic C-H in-plane de-formation and C-O deformation of primary alcohol, and a band at 1085 cm^{-1} originating mainly from C-O deformation (Hergert, 1971). The increasing intensity of the 1085 cm^{-1} band from MS EFR to R3 reflected the enrichment of the successive alkali insoluble residues in cellulose, glycosidic linkages contributing to the C-O signal. Likewise, this band was found proportionally stronger for LC2 than for LC1, in agreement with the higher carbohydrate content of LC2. The higher carbohydrate content of LC2 was also reflected by the stronger relative intensity of the band at 900 cm^{-1}, corresponding to the carbohydrate C_1-H deformation and ring-stretching frequency (Stewart and Morrison, 1992). Besides signals characteristic of carbohydrates, the MS EFR spectrum exhibited a band at 1735 cm^{-1} corresponding to unconjugated carbonyl stretch (Faix, 1991). Because only trace amounts of uronic acids were detected in these samples, this band could be assigned to the carbonyl group carried by FA esters in feruloylated AXs and to CA esters in lignins. Accordingly, the relative intensity of this band was found to decrease from MS EFR to R1 and again slightly from R2 to R3, in agree-

ment with the removal of arabinoxylans and esterified FA and CA as a consequence of the alkali extractions. Lignin signals were detected in the 1100-1600 cm^{-1} region. A broad band at 1242 cm^{-1} in the spectra of MS EFR was assigned to stretching of C-O linked to syringyl or guaiacyl units of lignin (Hergert, 1971). Its absence from the spectra of the insoluble residues (R1-R3) reflected selective removal of lignin at each extraction step. The bands at 1420 cm^{-1}, 1460 cm^{-1}, and 1510 cm^{-1} were attributable to aromatic skeletal vibrations of G-S lignin (Hergert, 1971). In agreement with the decreasing lignin content, the band at 1510 cm^{-1} (indicated by arrows in Fig. 2B) was found relatively stronger in the spectra of MS EFR and LC1 than in the spectra of other fractions where it was hardly detected. Besides lignin units, hydroxycinnamic acids were also expected to contribute to this aromatic band (Stewart and Morrison, 1992). Indeed, comparison of spectra of LC2 and MS EFR suggests that CA and FA gave marked contribution at 1510 cm^{-1} because at comparable lignin contents, the 1510 cm^{-1} band was less pronounced for LC2. A closer look at the spectra allowed also detecting changes specific to lignin. The band at 1166 cm^{-1}, typical for HGS lignins (Faix, 1991), was present in each spectrum but was found the weakest in LC1, with a more flattened shape compared to LC2. This characteristic was tentatively assigned to the less condensed structure of lignin in LC1 (Table 4). Thus, the observed changes in the spectra were found to be consistent with the compositional changes evidenced by chemical analysis. This result confirms that ATR-FTIR is a high-throughput and convenient tool to monitor how the fractionation process affected lignocellulosics.

4 Conclusions

Based on the sequential fractionation of maize stem extractive-free material leading to recovery of 47% total lignin, the occurrence of two major types of lignins in maize stem is proposed. Lignins soluble in dilute alkali at room temperature are enriched in non-condensed inter-linkages in contrast to the second type of lignins that are nearly entirely condensed and more closely associated with cellulose. A portion of this second type of lignins can be co-solubilized with carbohydrate in alkali from pre-extracted residue hydrolyzed with endoglucanase. The distinct lignin structure and carbohydrate composition very likely impact different properties of the isolated lignin-carbohydrate fractions.

Acknowledgements

The authors thank Alexandre Cukier and Laurent Cézard for technical assistance with mass spectrometry, Frédéric Legée for skillful assistance in preparation of the MS EFR, and Sylvie Citerne for assistance with ATR-FTIR. M.H.S. acknowledges grants from Foundation of Emil Aaltonen and Finnish Foundation for Technology Promotion.

5 References

1. Adler, E. Lignin Chemistry-Past, Present and Future., 1977. Wood Sci. Technol. 11, 169-218.

2. Beckman, E., Liesche, O., Lehman, F., 1923. Qualitative und quantitative Unterschiede der Lignin einiger Holz- und Stroharten. Biochem. Z. 139, 491-508.

3. Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A., 1983. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydr. Res. 113, 291-299.

4. Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72, 248-254.

5. Charles, M.B., Ryan, R., Ryan, N., Oloruntoba, R., 2007. Public policy and biofuels: The way forward? Energy Policy 35, 5737-5746.

6. Culhaoglu, T., Zheng, D., Méchin, V., Baumberger, S., 2011. Adaptation of the Carrez procedure for the purification of ferulic and p-coumaric acids released from lignocellulosic biomass prior to LC/MS analysis. J. Chromatogr. B 879, 3017-3022.

7. Dence, C. W., 1992. The determination of lignin, in: Lin, S. Y. and Dence, C. W. (Eds.), Methods in Lignin Chemistry. Springer-Verlag, New York, pp. 33-61.

8. Domac, J., Richards, K., Risovic, S., 2005. Socio-economic drivers in implementing bioenergy projects. 28, 97-106.

9. Faix, B.O., 1991. Classification of lignins from different botanical origins by FT-IR spectroscopy. Holzforschung 45, S21-S27.

10. Fengel, D., Wegener, G., 1983. Wood: Chemistry, Ultrastructure, Reactions. de Gruyter, New York. pp. 225–239.

11. Gellerstedt, G. and Henriksson, G., 2008. Lignins: Major Sources, Structure and Properties. In: Monomers, Polymers and Composites from Renewable Resources, Eds. M.N. Belgacem and A. Gandini. Elsevier, Amsterdam, pp. 201-224.

12. Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257-268.

13. Grabber, J.H., Ralph, J., Hatfield, R.D., 2000. Cross-Linking of Maize Walls by Ferulate Dimerization and Incorporation into Lignin. J. Agric. Food Chem. 51, 4984-4989.

14. Hergert, H.L., 1971. Infrared spectra, in: Sarkanen, K.V., Ludwig, C.H. (Eds.), Lignins: Occurrence, Formation, Structure and Reactions. Wiley Interscience, New York, pp. 267-297.

15. Ibarra, D., Köpcke, V., Larsson, P.T., Jääskeläinen, A.-S., Ek, M., 2010. Combination of alkaline and enzymatic treatments as a process for upgrading sisal paper-grade pulp to dissolving-grade pulp. Biores. Technol. 101, 7416-7423.

16. Jackson, M.G., 1977. The alkali treatment of straws. Anim. Feed Sci. Technol. 2, 105-130.

17. Lapierre, C., Jouin, D. and Monties, B., 1989. On the molecular origin of the alkali solubility of gramineae lignins. Phytochemistry 28, 1401-1403.

18. Lapierre, C., Pollet, B., Rolando, R., 1995. New insights into the molecular architecture of hardwood lignins by chemical degradation methods. Res. Chem. Intermed. 21, 397-412.

19. Lapierre, C., Pollet, B., Ralet, M.-C., Saulnier, L., 2001. The phenolic fraction of maize bran: evidence for lignin-heteroxylan association. Phytochemistry 57, 765-772.

20. Lapierre, C., 2010. Determining lignin structure by chemical degradations, in: Heitner, C., Dimmel, D. and Schmidt, J.A. (Eds.), Lignin and lignans - Advances in Chemistry. CRC Press, Taylor & Francis Group, Boca Raton, pp. 11-48.

21. Lawoko, M., 2005. Lignin polysaccharide networks in softwoods and chemical pulps, characterization, structure and reactivity. Doctoral dissertation, Kungliga Tekniska högskolan (KTH), Stockholm, 64 p.

22. Lawoko, M., Henriksson, G., Gellerstedt, G., 2006. Structural Differences between the Lignin-Carbohydrate Complexes Present in Wood and in Chemical Pulps. Biomacromolecules 6, 3467-3473.

23. Lawther, J.M., Sun, R.-C., 1996. The fractional characterization of polysaccharides and lignin components in alkaline treated and atmospheric refined wheat straw. Ind. Crops Prod. 5, 87-95.

24. Lawther, J.M., Sun, R.-C., Banks, W.B., 1996. Fractional characterization of alkali-labile lignin and alkali-insoluble lignin from wheat straw. Ind. Crops Prod. 5, 291-300.

25. Li, Q., Gao, Y., Wang, H., Li, B., Liu, C., Yu, G., Mu, X., 2012. Comparison of different alkali-based pretreatments of corn stover for improving enzymatic saccharification. Biores. Technol. 125, 193-199.

26. Méchin, V., Argillier, O., Menanteau, V., Barrière, Y., Mila, I., Pollet, B., Lapierre, C., 2000. Relationship of cell wall composition to in vitro cell wall digestibility of maize inbred line stems. J. Sci. Food Agric. 80, 574-580.

27. Miller, G.L., 1959. Use of DinitrosaIicyIic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426-248.

28. Nakamura, S., Wakabayashi, K., Nakai, R., Aono, R., Horikoshi, K., 1993. Purification and Some Properties of an Alkaline Xylanase from Alkaliphilic Bacillus sp. Strain 41M-1. Appl. Environ. Microbiol. 59, 2311-2316.

29. Ralph, J., 2010. Hydroxycinnamates in lignification. Phytochem. Rev. 9, 65-83.

30. Sipponen, M., Pastinen, O.A., Strengell, R., Hyötyläinen, J.M.I., Heiskanen, I.T., Laakso, S., 2010. Increased Water Resistance of CTMP Fibers by Oat (Avena sativa L.) Husk Lignin. Biomacromolecules 11, 3511-3518.

31. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2011. Determination of structural carbohydrates and lignin in biomass, Laboratory Analytical Procedure (LAP), NREL/TP-510-42618, Revised July 2011.

32. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. Laboratory Analytical Procedure (LAP), NREL/TP-510-42623, January 2008.

33. Stewart, D., Morrison, I.M., 1992. FT-IR Spectroscopy as a Tool for the Study of Biological and Chemical Treatments of Barley Straw. J. Sci. Food Agric. 60, 431-436.

34. Willför, S., Pranovich, A., Tamminen, T., Puls, J., Laine, C., Suurnäkki, A., Saake, B., Uotila, K., Simolin, H., Hemming, J., Holmblom, B., 2009. Carbohydrate analysis of plant materials with uronic acid-containing polysaccharides–A comparison between different hydrolysis and subsequent chromatographic analytical techniques. Ind. Crops. Prod. 29, 571-580.

35. Zhang, Y., Culhaoglu, T., Pollet, B., Melin, C., Denoue, D., Barrière, Y., Baumberger, S., Méchin, V., 2011. Impact of Lignin Structure and Cell Wall Reticulation on Maize Cell Wall Degradability. J. Agric. Food Chem. 59, 10129-10135.

Figure captions

Figure 1. Scheme for fractionation of maize stem EFR into lignin-carbohydrate fractions LC1, LC2 and alkali-insoluble residues R1-R3.

Table 1. Dry matter (DM) and Klason lignin (KL) yields of the main fractions recovered from the sequential fractionation procedure shown in Fig.1.

Yield relative to EFR (%)[a]		
Fraction	DM	KL
MS EFR	100	100
LC1	17.4	38.5
LC2	7.62	8.23
R3	42.3	18.9
Total recovered	67.3	65.6
Difference	32.7	34.4

[a]: Sampling of R1 and R2 for characterization is considered in the calculations.

Table 2. Total carbohydrate (CH) content, monosaccharide composition, and Klason lignin content of the LCs and lignocellulosic residues obtained from fractionation of EFR. The values shown are averages of two replicates ± absolute deviations from the mean.

Fraction	Total CH	Composition (% of total CH)[a]				KL
	(% w/w)	Ara	Gal	Glc	Xyl	(% w/w)
MS EFR	63.2 ± 0.6	3.72	0.94	63.3	32.0	17.3 ± 0.1
R1	78.6 ± 0.3	3.77	0.73	74.3	21.2	8.29 ± 0.01
R2	80.8 ± 0.7	3.41	0.65	73.7	22.1	10.2 ± 0.3
R3	85.6 ± 0.8	2.17	0.36	88.4	9.05	7.71 ± 0.02
LC1	50.3 ± 1.3	10.5	ND[b]	6.44	83.0	38.3 ± 0.5
LC2	70.7 ± 1.2	5.92	ND	11.5[c]	82.6	18.7 ± 1.1

[a]: Calculated as anhydrous monosaccharides: Ara, arabinose; Gal, galactose, Glc, glucose, Xyl, xylose. [b]:ND – not detected. [c]:Determined by analysis of the KL filtrate. CH content of LC1 and LC2 was determined by 4% H2SO4 hydrolysis (1 h, 121°C). CH content of other fractions was determined using the two-stage H_2SO_4 hydrolysis.

Table 3. Carbohydrate (CH) released from R1 (% w/w anhydrosugars/DM) by Novozym 476 analyzed before and after post-hydrolysis of the enzymatic hydrolysate.

Anhydrosugars (% w/w)	Ara	Gal	Glc	Xyl	Total
Monosaccharides[a]	0.02	0.00	3.09	0.08	3.19
Total carbohydrate[b]	0.32	0.11	4.69	0.67	5.79
Complex CH (%)[c]	94.8	99.8	34.2	88.4	45.3

Based on monosaccharide analysis with[a] and without[b] acid hydrolysis of the enzymatic hydrolysate. Samples were analyzed as analytical duplicates (absolute deviation from the mean was < 5%) and were corrected for monosaccharides released by self-hydrolysis or acid hydrolysis of the enzyme preparation.. [c]:Percentage of complex carbohydrate of the total amount.

Table 4. Thioacidolysis analyses of the LC and lignocellulosic residues obtained from the fractionation of maize stem EFR. The data are mean values from duplicate analyses ± absolute deviations from the mean.

Fraction	Total yield H+G+S (μmol g^{-1} lignin)	Relative proportion (% molar)			S/G molar ratio	% of units only involved in β-O-4 bonds[a]
		%H	%G	%S		
MS EFR	836 ± 6	1.3 ± 0.1	52.3 ± 0.6	46.4 ± 0.6	0.89 ± 0.02	16.7 ± 0.1
R1	768 ± 16	0.8 ± 0.2	56.2 ± 0.7	42.9 ± 0.5	0.76 ± 0.02	15.4 ± 0.3
R2	817 ± 72	1.0 ± 0.1	54.2 ± 0.5	44.7 ± 0.4	0.82 ± 0.02	16.3 ± 1.4
R3	575 ± 16	1.3 ± 0.0	61.7 ± 0.3	37.3 ± 0.2	0.61 ± 0.01	11.5 ± 0.3
LC1	1168 ± 8	1.4 ± 0.1	44.7 ± 0.6	53.9 ± 0.5	1.21 ± 0.03	23.4 ± 0.2
LC2	98 ± 6	0.7 ± 0.1	48.2 ± 0.8	51.1 ± 0.7	1.06 ± 0.03	2.0 ± 0.1

[a]: with the assumption that the average molar mass of a C_6C_3 grass lignin unit is 200 g mol^{-1}.

Table 5. Content of CA and FA (mg g^{-1}) in maize stem EFR and fractions sequentially isolated from it. Values shown are percentages averaged on two samples ± absolute deviations from the mean.

	CA[a]			FA[b]		
Fraction	Ester	Ether	Ester /ether	Ester	Ether	Ester /ether
MS EFR	20.3 ± 1.1	2.70 ± 0.42	7.52	6.45 ± 0.40	3.63 ± 0.23	1.77
R1	2.66 ± 0.03	2.11 ± 0.11	1.26	0.47 ± 0.24	1.26 ± 0.05	0.38
R2	2.32 ± 0.02	2.03 ± 0.09	1.14	0.27 ± 0.18	1.42 ± 0.17	0.19
R3	0.32 ± 0.06	1.94 ± 0.04	0.17	0.06 ± 0.06	1.37 ± 0.21	0.05
LC1	7.28 ± 0.33	8.23 ± 0.01	0.88	1.71 ± 0.14	12.3 ± 0.56	0.14
LC2	0.31 ± 0.02	2.80 ± 0.20	0.11	0.03 ± 0.03	2.73 ± 0.09	0.01

[a]:Sum of (*E)* and (*Z)* isomers; [b]*(E)* isomer.

Table 6. Mass balance showing fractionation of CA and FA into the two lignin-carbohydrate fractions and final insoluble residue R3. Total yields of FA and CA as well as ether-linked CA and FA are given relative to the starting maize stem EFR material.

Yield relative to EFR (%)[a]	Total		Ester		Ether	
	CA	FA	CA	FA	CA	FA
MS EFR	100	100	100	100	100	100
LC1	11.7	24.2	6.24	4.61	53.0	58.9
LC2	1.03	2.09	0.12	0.04	7.90	5.73
R3	4.16	6.00	0.67	0.39	30.4	16.0
Total recovered (%)	16.9	32.3	7.02	5.04	91.3	80.6
Difference (%)	83.1	67.7	93.0	95.0	8.7	19.4

[a]: Yields have been calculated using mean values of CA and FA given in Table 5 and DM yields given in Table 1.

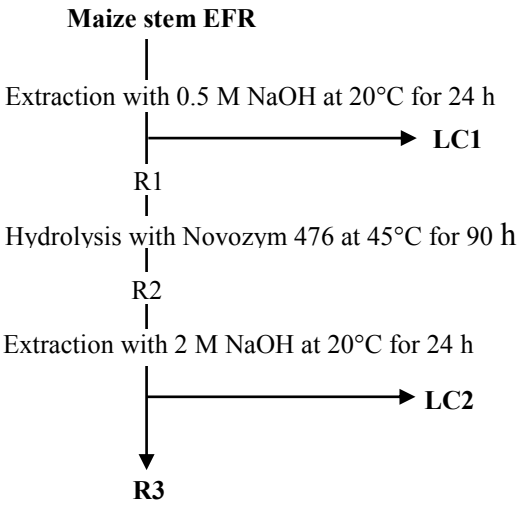
Maize stem EFR
Extraction with 0.5 M NaOH at 20°C for 24 h
LC1
R1
Hydrolysis with Novozym 476 at 45°C for 90 h
R2
Extraction with 2 M NaOH at 20°C for 24 h
LC2
R3

Figure 1